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Involvement of insulin-like growth factor binding protein-3 in peroxisome proliferator-activated receptor gamma-mediated inhibition of breast cancer cell growth

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ABSTRACT

We have previously reported that insulin-like growth factor binding protein-3 (IGFBP-3), a protein with dichotomous effects on both cell proliferation and cell survival, interacts with peroxisome proliferator-activated receptor gamma (PPARγ) and inhibits adipogenic PPARγ signaling. We now show that IGFBP-3 and PPARγ interact in breast cancer cells, through amino- and carboxyl-terminal residues of IGFBP-3. IGFBP-3 and the PPARγ ligands, rosiglitazone or 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2, separately inhibited the proliferation of MCF-7, MDA-MB-231 and MDA-MB-468 breast cancer cells. However, growth inhibition by IGFBP-3 and PPARγ ligand combined was greater than by either alone. Two IGFBP-3 mutants with reduced PPARγ binding caused no growth inhibition when used alone and abolished the inhibitory effect of rosiglitazone when used in combination with PPARγ ligand. Cell growth inhibition by PPARγ ligands was substantially blocked by IGFBP-3 siRNA and restored by exogenous IGFBP-3. We conclude that the interaction between IGFBP-3 and PPARγ is important for the growth-inhibitory effect of PPARγ ligands in human breast cancer cells, suggesting that IGFBP-3 expression by breast tumors may regulate their sensitivity toward PPARγ ligands.

1. Introduction

Insulin-like growth factor (IGF) binding protein-3 (IGFBP-3), one of six IGFBPs, binds IGF-I and -II with high affinity, inhibiting their cellular effects mediated by IGF-I receptor activation (Firth and Baxter, 2002; Yamada and Lee, 2009). IGFBP-3 also has dichotomous effects on breast cancer cell proliferation and apoptosis, independent of IGF signaling (Baxter, 2014). For example, IGFBP-3 contributes to the actions of a variety of pro-apoptotic stimuli (Brosseau et al., 2013; Butt et al., 2000; Perks et al., 2011), but exerts proliferative and pro-survival effects in breast cancer cells by potentiating growth factor signaling through sphingosine kinase activation (Martin et al., 2014) and stimulating autophagy by binding to glucose regulated protein 78 (Grkovic et al., 2013).

IGFBP-3 has a functional nuclear localization signal and can be translocated to the cell nucleus by binding to importin β (Schedlich et al., 2000). IGFBP-3 is a binding partner of type II nuclear hormone receptors (Yamada and Lee, 2009): retinoic acid receptor-α (RARα) (Liu et al., 2000; Schedlich et al., 2004), vitamin D receptor (VDR) (Schedlich et al., 2007b), nur77 (Lee et al., 2005), peroxisome proliferator-activated receptor-γ (PPARγ) (Chan et al., 2009) and their heterodimerization partner, retinoic X receptor-α (RXRα) (Liu et al., 2000). IGFBP-3 interferes with the heterodimer-mediated signaling of RARα:RXRα, blocking the cell's response to retinoic acid (Liu et al., 2000). When IGFBP-3 levels are immunodepleted in all-*trans*-retinoic acid (atRA)-resistant breast cancer cells, the cells become re-sensitized to the growth-inhibitory effects of atRA (Schedlich et al., 2004). Furthermore, non-retinoid receptor binding mutants of IGFBP-3 fail to block the growth inhibitory effects of atRA in human osteosarcoma cells, suggesting that IGFBP-3 binding to retinoid receptors is required for RAR-signaling modulation (Schedlich et al., 2007a). In contrast, IGFBP-3 enhances RXR response element-mediated signaling (Liu et al., 2000) and acts synergistically with RXRα to induce apoptosis in prostate cancer cells (Liu et al., 2005).

PPARγ is a ligand-dependent transcription factor with an essential role in adipogenesis and fat cell metabolism (Tontonoz and Spiegelman, 2008). We previously showed that IGFBP-3 inhibits preadipocyte differentiation into adipocytes, a PPARγ-dependent process, and proposed that IGFBP-3 inhibits adipogenesis at least in part by binding to PPARγ, since endogenous IGFBP-3 and PPARγ are binding partners in 3T3-L1 adipocytes (Chan et al., 2009). The

Abbreviations: IGF, insulin-like growth factor; IGFBP-3, IGF binding protein-3; RARα, retinoic acid receptor-α; VDR, vitamin D receptor; PPARγ, peroxisome proliferator-activated receptor-γ; RXRα, retinoic X receptor-α; atRA, all-*trans*-retinoic acid; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; TZD, thiazolidinedione.

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arachidonate metabolite 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is a natural ligand of PPARγ capable of activating reporter constructs containing PPARγ response elements and stimulating PPARγ-dependent adipogenesis (Forman et al., 1995; Kliewer et al., 1995). Thiazolidinedione (TZD) drugs, including rosiglitazone and ciglitazone, are synthetic ligands of PPARγ that were designed to treat type-2 diabetes mellitus by reducing insulin resistance of peripheral tissues (Nolan et al., 1994). Antiproliferative effects of PPARγ ligands in breast cancer cells have been demonstrated (Elstner et al., 1998; Hatton and Yee, 2008; Kim et al., 2006), but these are not always PPARγ-dependent (Lecomte et al., 2008; Turturro et al., 2004).

We hypothesized that the action of PPARγ ligands as antiproliferative agents in breast cancer may be modulated by the interaction of PPARγ with IGFBP-3. This is consistent with our analysis of publicly available transcriptomic data showing that survival is increased among women with breast tumors expressing high PPARγ as well as high IGFBP-3 levels. We have identified IGFBP-3 residues that are required for this interaction and shown that inhibition of cellular proliferation and anchorage-independent growth by IGFBP-3 and PPARγ in combination was greater than for either treatment alone. These results suggest that breast tumors that express IGFBP-3 may show enhanced sensitivity to PPARγ ligands.

2. Materials and methods

2.1. Materials

Rosiglitazone and 15d-PGJ_2 were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The PPARγ antagonist, GW9662, was purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). The pRL-TK and pRL-CMV constructs were purchased from Promega (Madison, WI, USA). Recombinant human IGFBP-3 (rhIGFBP-3) and mutant proteins were purified from the conditioned media of 911 human embryonic retina cells infected with adenoviral expression vectors as previously described (Firth et al., 1998; Schedlich et al., 2007a; Yan et al., 2004).

2.2. Cell culture and transfections

The human breast cancer cell lines used in the study were originally obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). MDA-MB231 and MDA-MB-468 cells were grown from laboratory stocks that were within two passages after receipt from ATCC. MCF-7 cells were authenticated using short tandem repeat profiling (CellBank Australia, Westmead, NSW, Australia). Experiments were typically conducted with cells that had undergone less than 30 passages. Cells were maintained in RPMI 1640 medium (Thermo Scientific, Waltham, MA, USA) supplemented with 20 mM Hepes, 10% v/v FBS, 2 mM L-glutamine and 10 μg/mL bovine insulin in 5% CO_2/95% air at 37 °C in a humidified incubator. Serum-free media containing RPMI 1640 medium was supplemented with 20 mM Hepes, 2 mM L-glutamine, 10 μg/mL bovine insulin and 0.1% w/v bovine serum albumin. For transient transfections, cells were electroporated with siRNA using Nucleofector Kit V and the Amaxa Nucleofector (Lonza, Basel, Switzerland) according to the manufacturer's protocols. Cells were immediately transferred to medium and seeded into culture dishes.

2.3. GST pull-down assay

GST-PPARγ fusion proteins, expressed in *Escherichia coli* and immobilized on glutathione Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, UK), were incubated with 100 ng of rhIGFBP-3 as previously described (Schedlich et al., 2007a).

2.4. Western immunoblotting and radioimmunoassay

For immunoblotting, proteins separated by SDS-PAGE were transferred to nitrocellulose membranes (GE Healthcare) which were blocked in 5% w/v skim milk powder in TBST (0.1 M Tris, 1.2 M NaCl, pH 7.4 and 0.1% v/v Tween 20) for 2 h at RT, then incubated with primary antibody. After washes with TBST, blots were incubated with secondary antibody conjugated to horseradish peroxidase, washed again, and incubated with ECL Plus Western Blotting Detection Reagent (GE Healthcare). Chemiluminescent signals were detected using the LAS-3000 system and quantitated using Multi-Gauge 3.11 software (Fujifilm, Brookvale, NSW, Australia). Secreted IGFBP-3 levels in culture media were measured by radioimmunoassay (RIA) as previously described (Baxter and Martin, 1986).

2.5. Quantitative real time RT-PCR

RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's instructions and the concentration of RNA was quantitated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription was performed with 2 μg of total RNA using the SuperScript III First-Strand Synthesis Supermix kit (Life Technologies) and qRT-PCR was performed in a AB7900HT RT-PCR system (Life Technologies), according to the manufacturer's instructions. The following Taqman probes were used for cDNA amplification of the housekeeping gene hydroxymethylbilane synthase (HMBS, Assay ID: Hs00609297_m1), IGFBP-3 (Assay ID: Hs00181211_m1) and PPARγ (Assay ID: Hs00234592_m1).

2.6. Cell proliferation and colony formation assays

To measure cell proliferation, cells in standard RPMI 1640 medium with 1% v/v FBS were seeded at 5×10^4 cells/well in 24-well plates. After overnight culture, media were changed to serum-free medium (RPMI 1640 medium containing 0.1% v/v BSA) for 24 h prior to treatment. Media were replaced with standard RPMI 1640 medium containing 1% FCS with or without treatments, as indicated, for a further 72 h. Cells were then trypsinized and viable cells determined by Trypan blue (Sigma-Aldrich) exclusion.

The colony formation assay was performed in six-well plates with a base layer containing 0.75% w/v SeaPlaque agarose (Karlan, Cottonwood, AZ, USA) in complete growth medium (RPMI 1640 supplemented with 5% v/v FBS, 20 mM Hepes, 2 mM L-glutamine, 10 μg/mL bovine insulin and relevant treatments), a middle layer containing 1×10^4 cells resuspended in 0.5% w/v agarose in complete growth media, and a third layer of 0.5% w/v agarose in complete media. Finally 1 mL of complete growth medium containing relevant treatments was added to each well. Colonies were allowed to form over 2 weeks with medium changed after 7 days, then stained with 1 mg/mL p-iodonitrotetrazolium violet dissolved in PBS and incubated at 37 °C for 24–36 h. Plates were photographed using the LAS-3000 system and colonies were counted using the Colony software version 1.1 (Fujifilm).

2.7. Statistical analysis

Experiments were performed three times in triplicate unless otherwise stated. Statistical analysis (StatView for Windows v5.0, SAS Institute Inc, Cary, NC, USA) was performed using ANOVA with post-hoc analysis by Fisher's protected least significant difference (PLSD) test. Results were considered significant if $P < 0.05$. Data are expressed as mean ± SEM.